

Cell cycle checkpoint signaling involved in histone deacetylase inhibition and radiation-induced cell death

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Abbreviations List: HDAC, histone deacetylase; Plk1, Polo-like kinase-1; TSA, trichostatin A; PARP, poly(ADP-ribose) polymerase-1; CDGE, constant denaturant gel electrophoresis; CI, combination index

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Abstract

In breast cancer radiation has a central role in the treatment of brain metastasis, even though tumor sensitivity might be limited. The tumor cell defense response to ionizing radiation involves activation of cell cycle checkpoint signaling. Histone deacetylase (HDAC) inhibitors, agents that cause hyperacetylation of histone proteins and thereby aberrations in the chromatin structure, may also override the DNA damage defense response and facilitate the radiation-induced mitotic cell death. In experimental metastasis models, the human breast carcinoma cell line MA-11 invariably disseminates to the central nervous system. We compared *in vitro* MA-11 cell cycle response profiles to ionizing radiation and HDAC inhibition. After radiation exposure the G₂/M phase accumulation and the preceding repression of the G₂ phase regulatory factors Polo-like kinase-1 and cyclin B1 required intact G₂ checkpoint signaling through the checkpoint kinase CHK1, whereas the similar phenotypic changes observed upon HDAC inhibition did not. The MA-11 cells did not show radiation-induced expression of the G₁ cell cycle inhibitor p21, indicative of a defective G₁ checkpoint and consistent with a point mutation detected in the tumor-suppressor *TP53* gene. Increase in the p21 level, however, was observed upon HDAC inhibition. Following pretreatment with the HDAC inhibitor, the efficiency of clonogenic regrowth after irradiation was reduced, which is in accordance with the concept of increased probability of mitotic cell death when the chromatin structure is disrupted. Among molecular cell cycle-targeted drugs currently in the pipeline for testing in early-phase clinical trials, HDAC inhibitors may have a therapeutic potential as radiosensitizers.

Introduction

In breast cancer radiation as therapeutic modality has clearly documented palliation effect on advanced metastatic disease in the brain or meninges. Within this patient population, however, survival may vary from a few months to a couple of years. Hence, improvement of the standard therapy might potentially benefit many patients (1).

We have previously characterized the MA-11 human breast carcinoma cell line for its ability to form experimental organ-specific metastases *in vivo*. This cell line was established from micrometastatic cells enriched from a bone marrow sample taken from a patient who was clinically devoid of metastatic disease (2), but was somewhat unexpectedly found to form metastases within the central nervous system after systemic injection in rodents (2–4). This experimental metastasis model has been characterized for therapeutic responses to a variety of pharmacological compounds with cytotoxic activity (3, 5).

Cell cycle checkpoints constitute regulatory mechanisms that do not allow the initiation of a new phase of the cell cycle to proceed before the previous one is completed (6). The tumor cell response to DNA damage involves a temporary cell cycle delay at the G₁/S or G₂/M boundaries, each based on unique mechanisms, to activate a cascade of responses to the damage, ultimately leading to the outcome of cell survival if the DNA is properly repaired, or, if not, to apoptotic or mitotic cell death (7).

The tumor-suppressor protein p53 is the primary regulator of the G₁ checkpoint (7). Essentially, in tumor cells with intact p53 function, DNA damage leads to rapid p53 stabilization by post-translational protein modifications as well as induction in the level of the G₁ phase inhibitor p21 (7).

In the orderly dividing cell the transition from the G₂ phase to mitosis is inhibited through phosphorylations of the Cdc2 kinase of the Cdc2/cyclin B complex. Upon the onset of mitosis these inhibitory phosphorylations are removed by the Cdc25C phosphatase. The activation of Cdc25C requires positive regulatory phosphorylation, accomplished by the Polo-like kinase-1 (Plk1) (8). DNA damage-induced G₂ checkpoint signaling, initiated by the ATM kinase and communicated through downstream mediator proteins like p53 and the checkpoint kinase CHK1 (6, 9), will ultimately disrupt the interaction of Cdc25C with Cdc2 (6). We have previously found that the mechanism of the G₂ phase response to ionizing radiation comprises repression of the genes for Plk1 and cyclin B1, *PLK* and *CCNB1* (10).

Molecular targeted agents can have direct effects on the cellular response pathways implicated upon exposure to ionizing radiation (11). A variety of pharmacological compounds, designed to target cell cycle regulatory mechanisms, have been shown to override the DNA damage defense response that prevents mitotic entry (9, 11). Hence, such agents may have a therapeutic potential as radiosensitizers by facilitating mitotic cell death, and several are currently tested in early-phase clinical trials. We have recently reported that pharmacological inhibition of the CHK1 kinase counteracted the tumor cell defense responses on *PLK* and *CCNB1*, and thereby the G₂ arrest, following radiation exposure (10, 12). In agreement with this, the concomitant treatment with the CHK1 inhibitor seemed to amplify the cytotoxic effect of ionizing radiation on clonogenic regrowth (12).

Drugs that modify the cellular chromatin structure may also radiosensitize tumor cells. Taxanes, which disrupt chromatin structure and chromosome segregation in mitosis, are currently utilized clinically as radiosensitizers in treatment of non-small cell lung cancer and head-and-neck cancer (13). Cellular treatment with histone

deacetylase (HDAC) inhibitors causes hyperacetylation of histone proteins, which leads to aberration in the chromatin structure (14). In addition to this, the perturbation by HDAC inhibitors of cell cycle checkpoint signaling (15), might constitute the cellular mechanism by which these compounds enhance tumor cell sensitivity to radiation treatment.

Currently several HDAC inhibitors are undergoing early-phase clinical investigation (14). Such pipeline drugs are not easily accessible, and in this report we have used a commercially available HDAC inhibitor, trichostatin A (TSA). TSA has shown excessive toxicity under *in vivo* conditions, and in this study we have therefore compared the biological mechanisms involved in the responses of the MA-11 cell cycle phenotype after exposure to TSA and ionizing radiation in cultured cells. A reduction in MA-11 clonogenic regrowth by the concomitant treatment with TSA and radiation is further indicated.

Materials and Methods

Cell Lines and Experimental Treatments

The human carcinoma cell lines MA-11 and MT-1 were cultured as previously described (4). High-energy radiation from a ^{60}Co source was delivered at a rate of approximately 1 Gy/min. The unirradiated control cells were simultaneously placed in room temperature to obtain exactly comparable conditions. The commercially available HDAC inhibitor TSA (Sigma-Aldrich Norway, Oslo, Norway) was added to the media in final concentrations of 10–300 nM. In experiments using the selective CHK1 kinase inhibitor UCN-01 (National Cancer Institute, Bethesda, MD), this compound was added in a final concentration of 100 nM to the cell media, as recommended by the supplier, 15 minutes before irradiation or TSA treatment.

Western Blot Analysis

Protein expression was measured by means of standard Western blot technique, essentially as previously described (10). The membranes were stained with amidoblack to evaluate equal protein loading, and subsequently hybridized with designated primary antibodies obtained from Upstate (Lake Placid, NY), Calbiochem/Merck Biosciences Ltd. (Nottingham, UK), Santa Cruz Biotechnology (Santa Cruz, CA), Chemicon International (Temecula, CA), or Zymed Laboratories Inc. (San Francisco, CA). These were anti-acetyl-histone H4 (Upstate; 06-866), anti- α -tubulin (Calbiochem; CP06), anti-poly(ADP-ribose) polymerase-1 (anti-PARP) (Calbiochem; 512739), anti-p53 (SC-6243), anti-MDM2 (Chemicon; MAB4134), anti-Plk1 (Zymed; 33-1700), and anti-p21 (SC-6246), respectively.

Flow Cytometry Analysis

The MA-11 cells were harvested in ice-cold PBS, and after centrifugation the cell pellets were fixed in 100% methanol. To determine the fractions of cells in the G₁, S, and G₂/M phases from the cell cycle distribution, the cells were stained with 1.5 μ g/ml Hoechst 33258 in PBS and analyzed in a FACStar+ flow cytometer (Becton Dickinson, San Jose, CA), as described previously (10).

Mutation Analysis of the *TP53* Gene

DNA extracted from the cell lines was analyzed for possible *TP53* mutations using the method of constant denaturant gel electrophoresis (CDGE) (16). The screening was performed using PCR to amplify exons 2, 3, and 6–11 individually, as well as the large exons 4 and 5 each as two sequential PCR products (17). The sample that showed aberrantly migrating bands, indicating mutation, was reamplified using the original set of primers, of which one was biotinylated, and the same thermal cycling condition. The biotinylated PCR product was sequenced directly by means of

the standard dideoxy method and Dynabeads M280–Streptavidin (Dyna, Oslo, Norway) as solid support.

Northern Blot Analysis

Expression of RNA was measured by means of standard Northern blot technique, as described previously (10). The human cDNA probe for *TP53* was provided by Dr. B. Smith-Sørensen (The Norwegian Radium Hospital, Oslo, Norway), whereas the human cDNA clones for *PLK* and *CCNB1* were obtained from RZPD Deutsches Ressourcenzentrum für Genomforschung GmbH (Berlin, Germany). The human cDNA probe for *CDKN1A* was a gift from Dr. B. Vogelstein (The Johns Hopkins University School of Medicine, Baltimore, MD). To evaluate the amounts of RNA loaded, the filters were rehybridized to a kinase-labeled oligonucleotide probe complementary to nucleotides 287-305 of human 18S rRNA.

Assessment of Clonogenic Regrowth

Clonogenic regrowth efficiency of the MA-11 cell line was determined by plating single cells suspended in media with or without TSA (10–300 nM) for 12 hours, before the media were replaced with fresh media and the cells irradiated. The appropriate plating density was aimed to produce 20–40 surviving colonies in each well of 6-well culture plates, and the mean plating efficiency of the control MA-11 cells was not more than ~0.225 in the three independent sets of experiments. After incubation for 2–3 weeks, the cells were fixed and stained with 0.1% crystal violet. Colonies of ≥ 50 cells were counted for computing of the surviving fraction. At least four parallel samples were scored in the three repetitions performed for each treatment condition.

Calculation of Radiation-TSA Interactions

The results from the clonogenic regrowth measurements were analyzed using the combination index (CI) method of Chou and Talalay (18). CI values <0.90 are indicative of synergistic interactions, whereas additive interactions are indicated by CI values of 0.90–1.0 and antagonistic interactions by CI values >1.1.

Results

HDAC Inhibition — Histone Acetylation and PARP Protein Status

Tumor cell sensitivity to pharmacological HDAC inhibition may vary along a wide concentration range and should be considered highly cell line specific. Thus, the initial experiment was performed to determine the effect of increasing concentrations of TSA (10–300 nM) on the histone acetylation status of the MA-11 cell line. As seen from Fig. 1, upper panel, the level of acetylated histone H4 was significantly induced after 6–12 hours exposure to the higher TSA concentrations (100 and 300 nM) before the level again dropped below detection after 24 hours. In contrast, histone H4 acetylation was not seen in cells treated with TSA in the lower concentration range (10 and 30 nM). The expression pattern of acetylated histone H3 was closely identical (data not shown), which might indicate that MA-11 cell histones are insensitive to TSA below a threshold concentration.

These data suggested that TSA in a concentration of 300 nM might be appropriate for further mechanistic studies. The possibility of MA-11 cell apoptosis by this high TSA concentration (19, 20) was analyzed by means of PARP cleavage as we have recently demonstrated that degradation of this nuclear repair enzyme is a sensitive indicator of apoptotic cell death activated by a *Pseudomonas* exotoxin A-containing immunotoxin in the MA-11 cells (21). In contrast to what was detected in the immunotoxin-treated cells, the PARP protein remained intact in MA-11 cells

incubated with 300 nM TSA throughout the observation period of 24 hours (Fig. 1, lower panel), excluding apoptosis as an essential mechanism in TSA-induced MA-11 cell death (see below).

Ionizing Radiation and HDAC Inhibition — Redistribution of Cell Cycle Phases

Pharmacological inhibition of HDAC activity has been shown to cause cell cycle arrest at the G₂/M boundary in a variety of tumor cell lines (19, 20, 22–24), resembling the G₂ checkpoint response to DNA damage induced by ionizing radiation (10, 12, 25). Hence, the MA-11 cells were exposed to a radiation dose of 8.0 Gy or 300 nM TSA, and cell cycle profiles were followed for 24 hours (Fig. 2, upper panel).

The irradiated cells displayed an apparent accumulation of G₂/M phase cells throughout the observation period. The fraction of G₂/M phase cells increased from 20–25% of the total cell counts after 12 hours to ~40% after 24 hours. A similar redistribution of cell cycle phases was observed in the TSA-treated cells, although a larger cell fraction was in S phase and a significantly lower fraction (~30% of the total cell counts) was arrested in the G₂/M phase after 24 hours, compared to cells exposed to ionizing radiation.

p53 Status

In the MA-11 cells a distinct G₁ phase was detected following both irradiation and TSA treatment (Fig. 2). The persisting G₁ phase cells may reflect an incomplete G₂/M phase arrest, allowing DNA-damaged cells to pass into the G₁ phase of a new cell cycle. Alternatively, the regulatory mechanism of such pattern of cell cycle redistribution may involve a functional G₁ checkpoint. Hence, the cellular p53 status was analyzed and compared with that of the MT-1 cell line, in which cell cycle responses to ionizing radiation have been reported previously (10, 12).

As depicted by Fig. 3, upper panel, CDGE analysis of DNA from the MA-11 cell line revealed aberrant migration of a PCR fragment representing exon 5 of the *TP53* gene, in accordance with the base substitution of C for a T nucleotide in codon 126, resulting in change of amino acid Tyr to His, detected by the subsequent sequencing of the PCR fragment. In contrast, we found with DNA from the MT-1 cells all exons of the *TP53* gene to be wildtype upon CDGE analysis.

Furthermore, both cell lines showed essentially equal levels of mRNA expression for *TP53* (Fig. 3, middle panel). However, whereas strong p53 protein expression was found in the MA-11 cell line, in accordance with the intense nuclear staining of p53 previously detected by immunocytochemistry (2), p53 expression was almost undetectable in the MT-1 cell extracts. A weak band representing the p53 protein was seen after long-term exposure of the immunoblot (Fig. 3, lower panel).

Primarily, p53 stabilization results from disruption of the interaction between p53 and the MDM2 oncoprotein, which thereby protects p53 from ubiquitin-mediated degradation (26). Whereas expression of two major MDM2 polypeptides was observed in the MT-1 cells, MDM2 was almost undetectable in the MA-11 cells (Fig. 3, lower panel).

Ionizing Radiation and HDAC Inhibition — Responses of Cell Cycle Regulatory Proteins

The responses of regulatory proteins of the G₁ and G₂ cell cycle phases were followed for 24 hours after MA-11 cell exposure to ionizing radiation (8.0 Gy) or TSA (300 nM). As seen from Fig. 4, expression of the G₂ phase kinase Plk1 was found to be down-regulated 6 hours after irradiation, with an apparent increase above the control Plk1 level, probably compensatory, after 12–24 hours. This has also been observed in irradiated MT-1 cells (12). A transient Plk1 repression was also seen

6–12 hours after start of the TSA treatment but almost recovered after 24 hours. From below detection, the level of the G₁ phase inhibitor p21 was induced 12 hours after addition of TSA and further observed as clearly accumulating after 24 hours. Coincident with this, the high level of p53 was significantly repressed. In contrast to the TSA-dependent effects on p21 and p53, the expression levels of these proteins were not altered by radiation.

The regulatory responses on cell cycle proteins seemed to reflect changes in mRNA levels after exposure of the MA-11 cells to ionizing radiation or TSA (Fig. 5). As previously observed in other breast cancer cell lines (10, 12, 25), the level of *PLK* mRNA (encoding Plk1) was barely detectable 6 hours after irradiation but almost recovered after 12 hours, and the response of *CCNB1* mRNA (encoding the G₂ phase-specific cyclin B1) was essentially identical. The transient down-regulation of these mRNAs by TSA was observed for some longer period (6–12 hours) before their expression again was up-regulated 24 hours after start of the TSA treatment. The mRNA expression of *CDKN1A* (encoding p21) was not altered after irradiation, which is highly indicative of a defective G₁ checkpoint (7). In the presence of TSA, however, expression of *CDKN1A* mRNA was inversely reflecting the regulatory effects on the mRNAs for *PLK* and *CCNB1*.

Ionizing Radiation and HDAC Inhibition — Regulatory Role of CHK1

We have recently shown that the cell cycle phenotype responses following radiation-induced DNA damage require intact G₂ checkpoint signaling through the downstream checkpoint kinase CHK1 (10, 12). In accordance with this, treatment with the CHK1 inhibitor UCN-01 (100 nM) also seemed to counteract the G₂/M phase arrest observed 12–24 hours after exposure of the MA-11 cells to a radiation dose of 8.0 Gy, but not upon incubation with TSA (300 nM) for 24 hours, as seen by

comparing the histograms displayed by the upper and lower panels of Fig. 2. Furthermore, whereas the suppressed mRNA levels of *PLK* and *CCNB1* following irradiation of the MA-11 cells were entirely abolished by UCN-01, the regulatory effects of TSA on those mRNAs, and on *CDKN1A* mRNA, were not (Fig. 5). Hence, our data strongly indicate that the effector mechanisms of the G₂ phase responses to ionizing radiation and HDAC inhibition are mediated via distinct regulatory pathways.

Ionizing Radiation and HDAC Inhibition — Clonogenic Regrowth

Finally, the MA-11 cell line was exposed to increasing doses of ionizing radiation to determine clonogenic survival (Fig. 6). The cell line showed nearly exponential loss of colony formation efficiency, with a surviving fraction of ~0.01 with the highest radiation dose applied (10 Gy).

Since the regulatory pathway recruited by HDAC inhibition seemed to be distinctly different from that following irradiation, the possible radiosensitizing effect of TSA, essentially by amplifying the cytotoxic effect of ionizing radiation on clonogenic regrowth, was measured. Based on the histone acetylation data (Fig. 1, upper panel), we chose to analyze MA-11 cells treated with TSA (10–300 nM) for 12 hours before the HDAC inhibitor was removed and the cells irradiated. This treatment strategy was supported by the observations that irradiation followed by the immediate TSA treatment for 12 hours did not reduce MA-11 cell clonogenicity compared to irradiation alone and that TSA incubations for 24 hours or more before or after radiation exposure turned out to induce complete cytotoxicity in this cell line (results not shown).

The effects of the TSA pretreatment on several radiation doses were measured (Fig. 6) and analyzed by means of the CI method (Table 1). In most of the dose range

analyzed (0.50–5.0 Gy) the ability of clonogenic regrowth of irradiated MA-11 cells was reduced by a factor of ≤ 3 after pretreatment with 300 nM TSA. The cytotoxic effect of 8.0 Gy of ionizing radiation on clonogenicity (surviving fraction of ~ 0.04), however, was ~ 10 -fold amplified by TSA (Fig. 6). Moreover, as depicted by Table 1, synergistic effects (CI values < 0.90) on MA-11 colony formation were found for most combinations of TSA and ionizing radiation tested, but, interestingly, TSA in the lower concentration range (10 and 30 nM) seemed to antagonize the cytotoxic effect of the 5.0 Gy radiation dose on the MA-11 cells.

Discussion

In this report we have compared cell cycle responses of the human breast carcinoma MA-11 cell line to ionizing radiation and HDAC inhibition. Whereas accumulation of G₂/M phase cells, as well as the preceding repression of the genes encoding the G₂ phase regulators Plk1 and cyclin B1, after irradiation required intact G₂ checkpoint signaling through the checkpoint kinase CHK1, the similar phenotypic changes observed upon HDAC inhibition did not. The MA-11 cells did not show radiation-dependent induction of the G₁ phase inhibitor p21, indicative of a defective G₁ checkpoint and possibly consistent with the base substitution detected in the tumor-suppressor *TP53* gene. Induction in the p21 level, however, was observed upon HDAC inhibition. Following pretreatment with the HDAC inhibitor, the efficiency of clonogenic regrowth after irradiation was reduced, which is in accordance with the concept of increased probability of mitotic cell death when the chromatin structure is disrupted.

Recent reports have shown that HDAC inhibitors by themselves possess antiproliferative effects in a variety of tumor cell lines (19, 20, 24). Moreover, some

potential of HDAC inhibitors to sensitize tumor cells to the DNA-damaging cytotoxicity of chemotherapeutics and ionizing radiation has lately been observed (27–32). Interestingly, in animal models the treatment with HDAC inhibitors significantly suppressed cutaneous side effects of radiation therapy (33), suggesting that the contemporary approach of molecularly targeted therapy may be utilized to increase the therapeutic ratio between the tumor and surrounding normal tissues in radiation therapy.

The clonogenic survival data clearly indicated that TSA in a wide concentration range may sensitize the MA-11 cells to the cytotoxic effect of ionizing radiation, even though a threshold concentration of the HDAC inhibitor seemed to be necessary to obtain histone acetylation, as measured by the expression of acetylated histones H4 and H3. It has been shown that TSA also acts via mechanisms involving hyperacetylation of non-histone proteins (22). Even though TSA seemed to cause acetylation of histones and non-histone proteins within the same concentration range, accumulation of acetylated non-histone proteins occurred more rapidly. This difference in TSA responses might be of consequence for its cellular toxicity (22) and perhaps account for the apparent antagonism observed by low concentrations of TSA in the MA-11 cells exposed to 5.0 Gy of ionizing radiation as well.

Pharmacological inhibition of HDAC activity has previously been shown to cause redistribution of cell cycle profiles resembling the G₂ checkpoint response to DNA damage induced by ionizing radiation (19, 20, 22–24). *PLK* and *CCNB1* are among several genes, encoding mitotic regulators, of which the mRNA expression levels are down-regulated following activation of the G₂ checkpoint (34). The present report is the first on TSA-directed down-regulation of *PLK* mRNA. Whether this is regulated at the level of transcription inhibition, similar to what has been shown for

the repressed *CCNB1* promoter activity upon HDAC inhibition (23, 35, 36), remains to be determined.

The promoter of the human *PLK* contains a characteristic repressor element in the region of the transcription start site, mediating the cell cycle phase-specific regulation of the gene expression (37). This repressor element is involved in the inhibition of *PLK* transcription after activation of p21 (38), which can function as a highly specific transcriptional regulator of numerous genes involved in cell cycle progression and DNA repair (39). However, the observation that *PLK* mRNA repression by TSA clearly preceded the induction of p21, argues against a p21-directed pathway as the principal effector mechanism.

Treatment with HDAC inhibitors induces p21 expression by a transcriptional mechanism, possibly mediated by the ATM kinase signaling pathway (40) and associated changes in the acetylation status of the *CDKN1A* promoter (23, 41). Accordingly, we found both *CDKN1A* mRNA and the p21 protein induced from very low baseline levels after addition of TSA. However, the effector mechanism of this p21 accumulation, supposed being initiated by ATM, did not seem to involve the downstream checkpoint kinase CHK1, which suggests diversity in the regulatory pathways governed by ATM in DNA damage checkpoint control. Similarly, our data on repression of Plk1 and cyclin B1 followed by G₂/M phase arrest after exposure to ionizing radiation and TSA strongly indicate that these G₂ phase responses, even if they are phenotypically similar, are mediated via distinct regulatory pathways.

In the MA-11 cells a distinct G₁ phase was detected following both irradiation and TSA treatment. The regulatory mechanism of such pattern of cell cycle redistribution might involve a functional G₁ checkpoint, in the event of which a radiation-induced up-regulation of *CDKN1A* mRNA should be observed (7).

However, the complete lack of such a response supports the assumption that the persisting G₁ phase instead reflects an incomplete G₂ phase arrest.

Consistent with a defective G₁ checkpoint, a base substitution in codon 126 of the *TP53* gene and a correspondingly high p53 protein level were detected in the MA-11 cell line. Primarily, p53 stabilization results from disruption of the interaction between p53 and the MDM2 oncoprotein, which thereby protects p53 from ubiquitin-mediated degradation (26). The expression of MDM2 was almost undetectable in the MA-11 cells. This is in accordance with the concept that tumor cells with high intrinsic levels of mutant, inactive p53 are unable to induce expression of the MDM2 protein, which would normally provide a feedback mechanism of p53 destabilization in the absence of DNA-damaging events (42).

Based upon the frequency of recorded *TP53* mutations (43), base substitutions in codon 126 are rare, but a few examples of missense mutations are reported, *e.g.*, in head-and-neck cancer (44, 45) and metastatic lesions from prostate cancer (46).

Basically, this report describes proof-of-principle experiments on how to use HDAC inhibitors to decrease the probability of clonogenic regrowth of tumor cells exposed to ionizing radiation. Although appealing as concept, caution must be shown upon interpretation and, indeed, possible therapeutic utilization. There are currently several HDAC inhibitors in early-phase clinical trials (14). It is reasonable to believe that therapeutic indications for these agents primarily will be in combination with conventional cytotoxic therapies.

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Table 1. CI values for ionizing radiation (IR) plus TSA

IR (Gy)	TSA (nM)	CI
2.0	10	0.67
2.0	30	0.67
2.0	100	0.51
2.0	300	0.51
5.0	10	1.2
5.0	30	1.1
5.0	100	0.88
5.0	300	0.73
8.0	10	0.78
8.0	30	0.77
8.0	100	0.43
8.0	300	0.29

Figure legends

Figure 1. HDAC inhibition by TSA in MA-11 cells — histone acetylation and PARP protein status. The cells were treated with TSA, either in increasing concentrations (upper panel) or in a concentration of 300 nM (+), or left untreated (–) (lower panel). Protein extracts prepared after 6–24 hours of incubation were analyzed by Western blot hybridization with an antibody against acetylated histone H4 (acetyl-H4) or an anti-PARP antibody that binds with higher affinity to the PARP cleavage fragment (85 kDa) than to the uncleaved fragment (116 kDa). The lower panel also includes a protein extract from MA-11 cells treated (+) with 10 ng/ml of a *Pseudomonas* exotoxin A-containing immunotoxin (IT), used as positive control for PARP cleavage (21). Expression of α -tubulin was measured as loading control.

Figure 2. MA-11 cell cycle profiles upon exposure to ionizing radiation (IR) or the HDAC inhibitor TSA. The cells were treated (+) with IR (8.0 Gy) or TSA (300 nM), or left untreated (–), in the absence (upper panel) or presence (lower panel) of the CHK1 inhibitor UCN-01 (100 nM), and further incubated for 12 and 24 hours before cellular DNA contents were determined by flow cytometry analysis gated for Hoechst 33258 fluorescence. Scales indicating cell counts (y axes) for G₁ and G₂/M phase cells, represented by the peaks labeled 2N and 4N, respectively, are provided.

Figure 3. p53 status of the MA-11 cells, as compared to that of the MT-1 cell line. Upper panel, exons 2–11 of the *TP53* gene were individually amplified by PCR from DNA extracted from the cell lines, and further analyzed by CDGE methodology. Any aberrantly migrating PCR fragments were subsequently sequenced. A detected single-

base mutation is indicated. wt, wildtype. Middle panel, *TP53* mRNA expression by the cell lines was analyzed by Northern blot hybridization, using 18S rRNA as RNA loading control. Lower panel, protein expression of p53 and MDM2 was determined by Western blot hybridization.

Figure 4. Checkpoint regulatory proteins in MA-11 cells after exposure to ionizing radiation (IR) or the HDAC inhibitor TSA. The cells were treated (+) with IR (8.0 Gy) or TSA (300 nM), or left untreated (–), and protein expression levels of Plk1, p21, p53, and acetylated histone H4 (acetyl-H4) after 0–24 hours were determined by Western blot analysis of cell extracts. Expression of α -tubulin was measured as loading control.

Figure 5. Cell cycle regulatory factors and CHK1 signaling after exposure of MA-11 cells to ionizing radiation (IR) or the HDAC inhibitor TSA. The cells were treated (+) with IR (8.0 Gy) or TSA (300 nM), or left untreated (–), in the absence (–) or presence (+) of the CHK1 inhibitor UCN-01 (100 nM). Expression levels of mRNAs for *PLK*, *CCNB1*, and *CDKN1A* after 0–24 hours were analyzed by Northern blot hybridization. 18S rRNA was measured as RNA loading control.

Figure 6. Inhibition of HDAC activity modulates the outcome of MA-11 clonogenic regrowth upon exposure to ionizing radiation. The MA-11 cells were exposed to increasing doses of ionizing radiation without (●) or following (○) pretreatment for 12 hours with TSA (300 nM), to determine relative colony formation compared to the unirradiated situation (mean \pm SEM, $n = 3$).

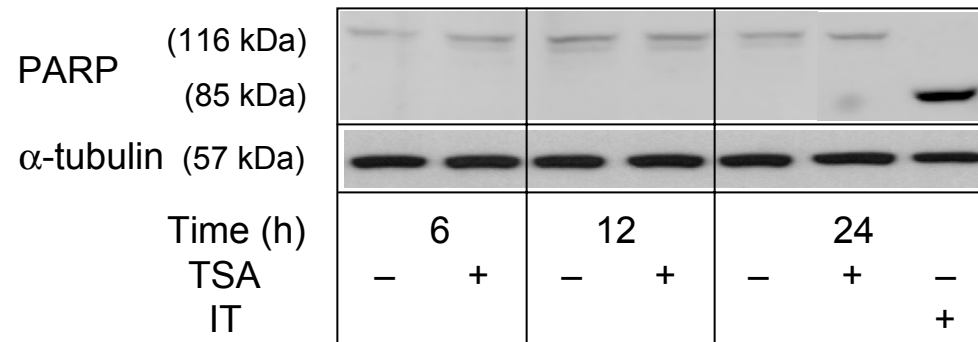
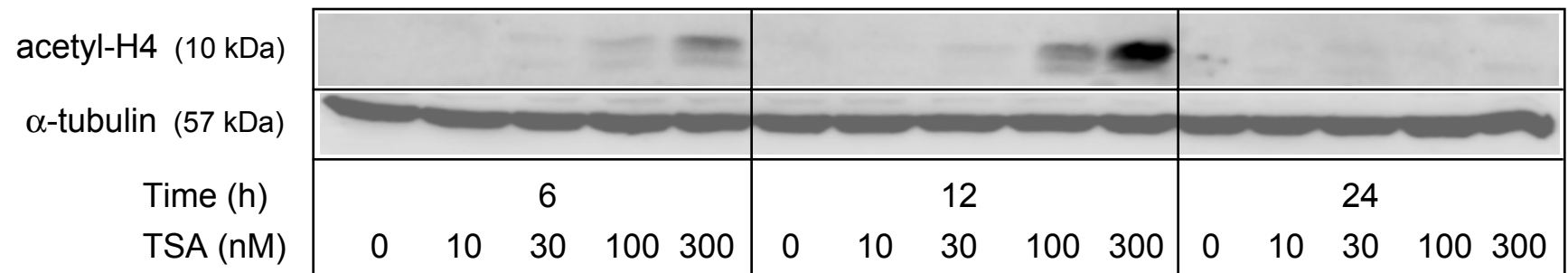


Figure 1

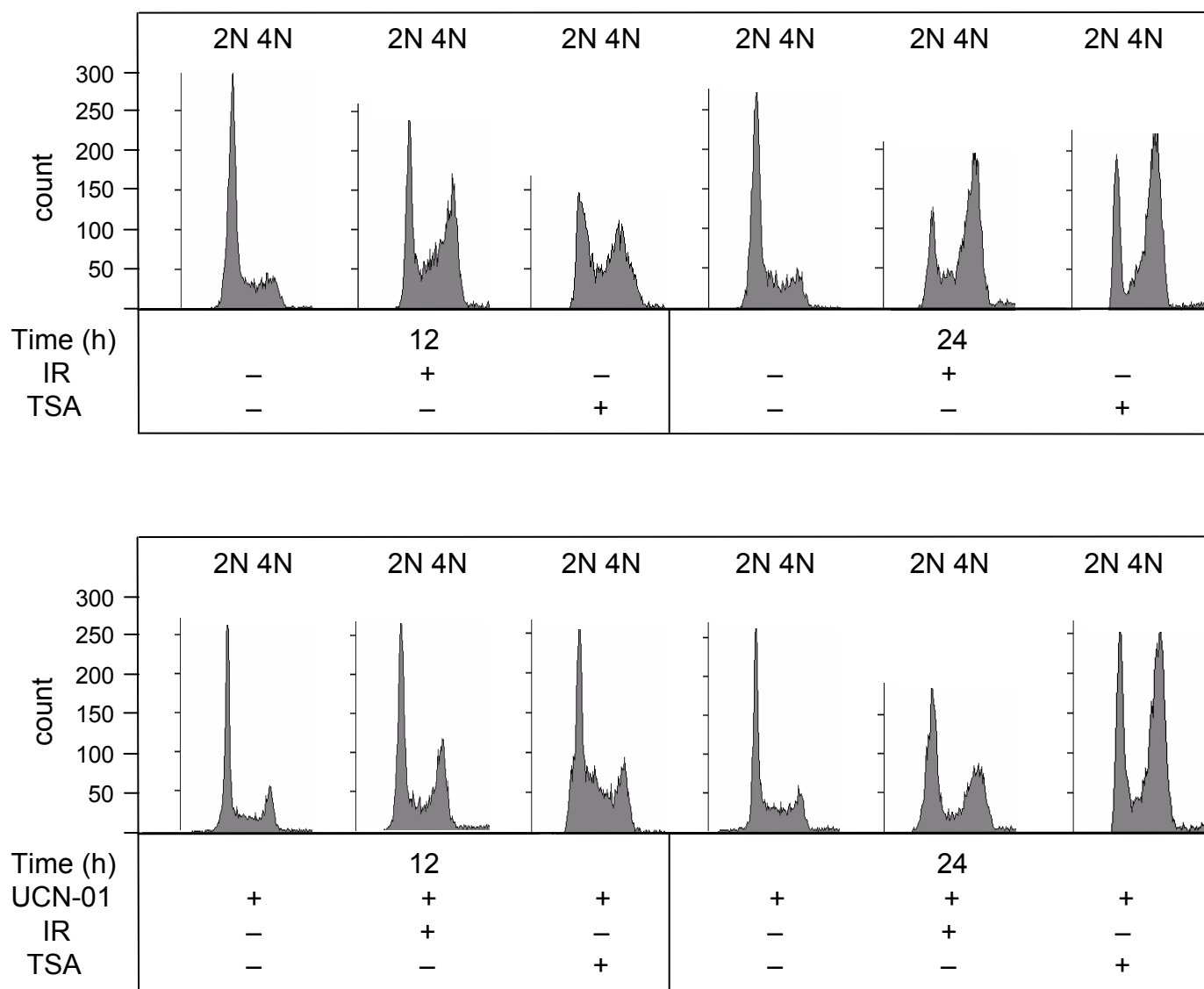


Figure 2

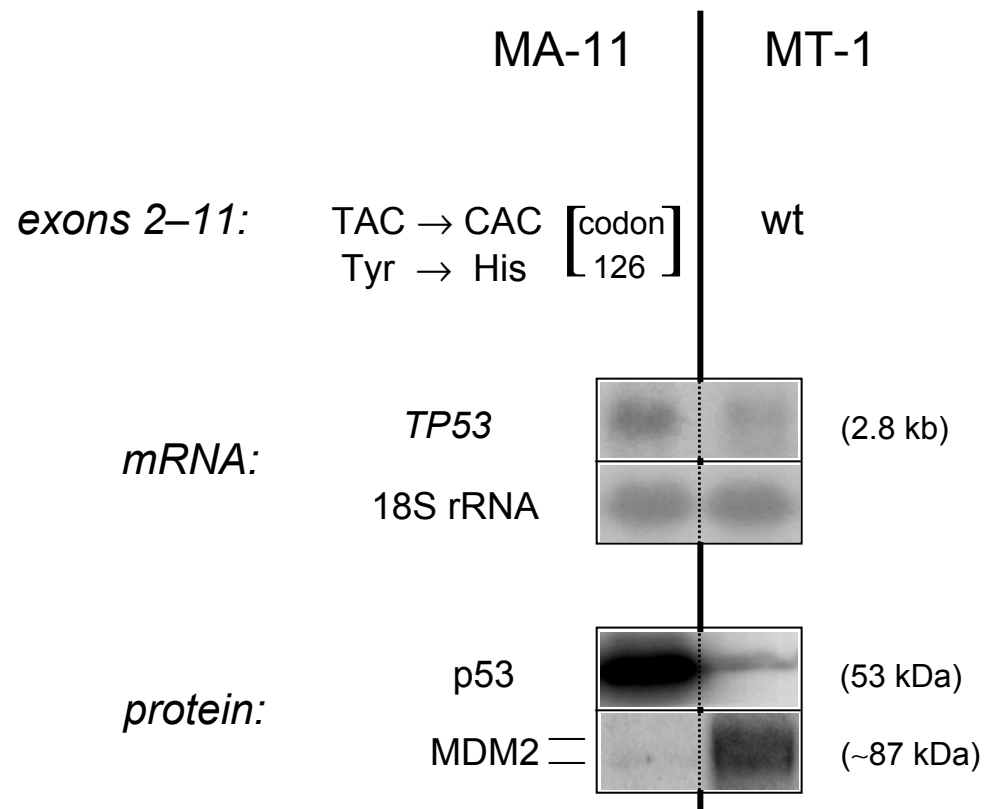


Figure 3

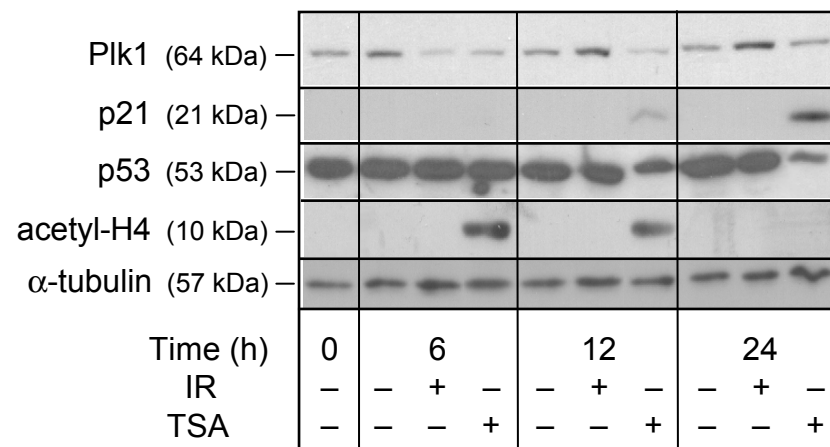


Figure 4

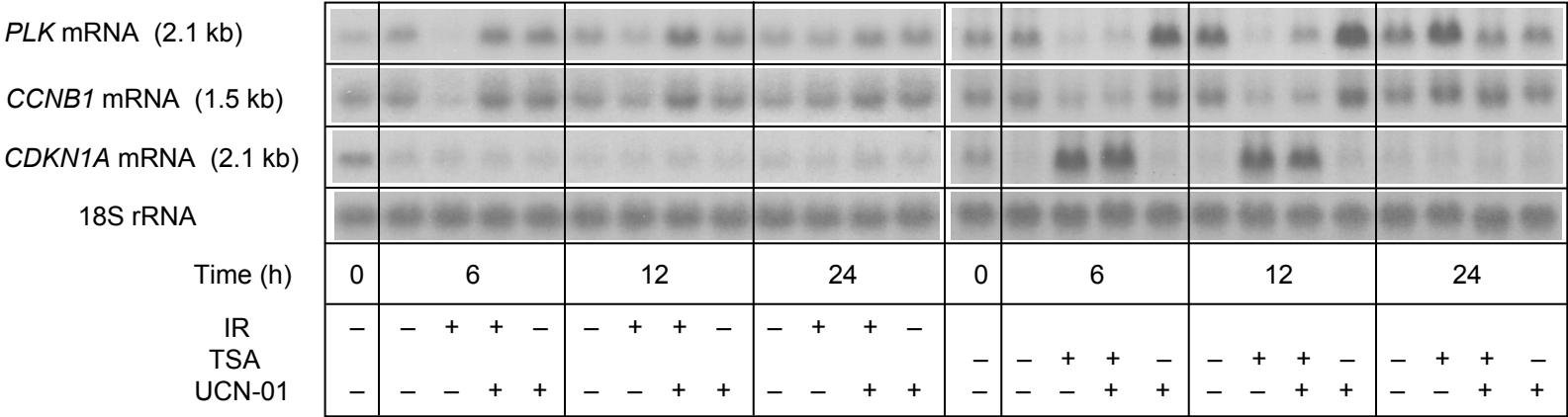


Figure 5

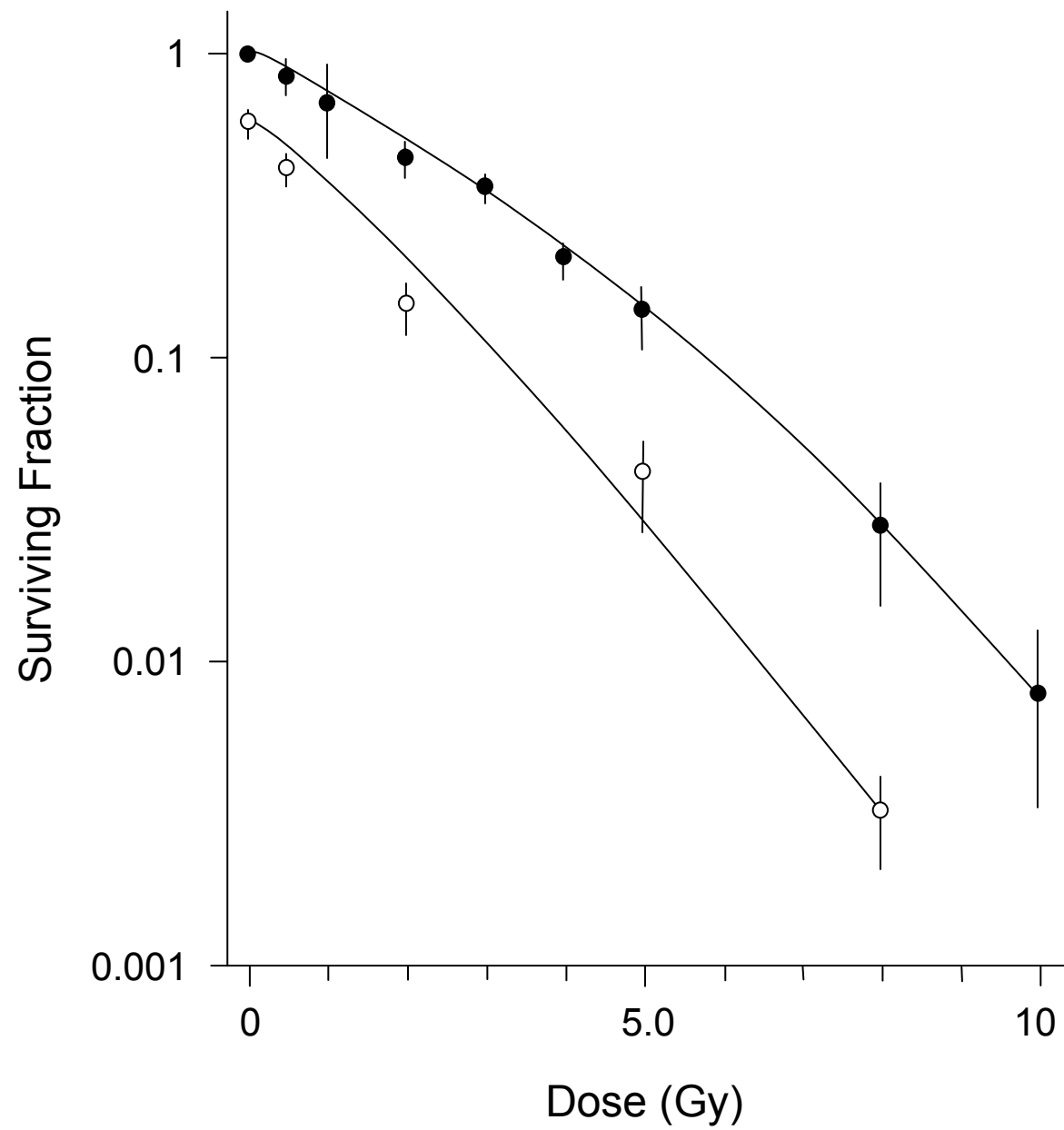


Figure 6